Changed

Primer R

↑ pases↑

GTGGCGAACGGGGGTCTTACCTCCCCACAGACATAA¥GACCCGCTCCACAGGA... CTTGCCCCCAGAATGGATGCGCATGTCTG CACCGCTTGCCCCCAGAATGGAGGGGGGGTGTCTG*T*ATTACTGGGCGAGGTGTCCT..

Target Sequence

**Polymorphic** nucleotide

PCR amplify

Fok I/Fsp J

CTTGCCCCCAGAATGGATGCGCATGT¢TGTATTACTGGGCGAGGTGTCCT.. GAACGGGGGTCTTACC<del>TCCTCCCACAC</del>ACATAATGACCCGCTCCACAGGA.

Digest with Fok I and Fsp I

S mer CTTGCCCCCAGAATGGATGC GCATGTCT GTATTACTGGGCGAGGTGTCCT.... cccacagacata atgacccgctccacagga.. GAACGGGGTCTTACCTCCT

Cut with Fok I

Fsp I

nnnnnnTGCGCAnnnnnn nnnnnACGCG‡nnnnn Cut with Fsp I

nnnnnnTGC GCAnnnnn nnnnnACG CGTnnnnn

Fok I GGATG CCGAC

TGCGCA Fsp.I.

Combined Fok I and Fsp I site

GGATGCGCA Fok I/Fsp I CCGACGCGT

........GTGGCGTTGCCCCCAGAATGGAGGAGGGTGTCTGTATTACTGGGCGAGGT... Fok I/Fsp I Loop Polymorphic Primer R1 G G A C CACCCCCAGAATGGAGGA GGTGTCCCCCAGAATGGAGGAGGAGGGTGTCCCCCAGAATGGAGGAGGGGGTGTCC Target Sequence

Deleted
base

PCR amplify

nucleotide

Fok I/Fsp I

GAACGGGGGTCTTACCTCCTCCTHCGCGTCCACHGACATAATGACCCGCTCCA... CTTGCCCCCAGAATGGAGGAGGATGCGCAGGT¢TCTG7ATTACTGGGCGAGGT

Digest with Fok I and Fsp I

Bsg I/Pvull Loop

TGGCTGGAGTTGCGCTAGCAAGA CAAAAGGATTTA

CGCCTATGGCTGGAGTTGCGCTAGCCAAGACCAAAAGGATTTATAAACTTC GCGGATACCGACCTCAACGCGATCGTTCTGGTTTTCCTAAATATTTGAAG ŝ

က်

PCR amplify

TGGCTGGAGTTGCGCTAGCAAGACGTGCAAAAGGGATTTATAAACTTC ACCGACCTCAACGCGATCGTTCTGCACGTCGACGTTTTCCTAAATATTTGAAGG Digest with Bsg I and Pvull

TGGCTGGAGTTGCGCTAGCAAGACGTGCAG CTGCAAAAGGATTTAT AAACTTC ACCGACCTCAACGCGATCGTTCTGCACGTC GACGTTTTCCTAAA TATTTGAAG

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က် Ω Pvull/Fok l Loop CGCCTATGGCTGGAGTTGCGCTAGCCAAGGACCAAAAGGATTTATAAACTTC GCGGATACCGACCTCAACGCGATCGTTCTGGTTTTCCTAAATATTTGAAG тевствсветнесеставсаавасса аавааттта PCR amplify

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CGCCTATGGCTGGAGTTGCGCTAGCAAGACCACAGGC†GGATGAAGGA†TTA7AAACTTC GCGGATACCGACCTCAACGCGATCGTTCTGGTGTCGACCTACTTCCTAAATATTTTGAAG

က်

Digest with Pvu II and Fok I

16 mer

CGCCTATGGCTGGAGTTGCGCTAGCAAGACCACAG CTGGATGAAGGATTTA TAAACTTC GCGGATACCGACCTCAACGCGATCGTTCTGGTGTC GACCTACTTCCTAAATATTT GAAG

Fok I/Fsp I

CTTGCCCCCAGAATGGAGGAGGATGCGCAGGTGTCTGTATTACTGGGCGAGGT... GAACGGGGGTCTTACCTCCTCCTACGCGTCCACAGACATAATGACCCGCTCCA...

Remove nucleotides and digest with Fok I

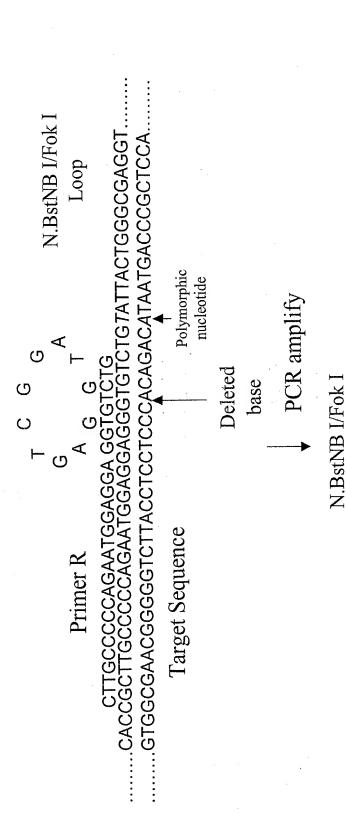
CTTGCCCCCAGAATGGAGGAGGATGCGCAGGTGT GAACGGGGGTCTTACCTCCTCCTACGCGTCCACAGACA

Fill in with mass

Modified nucleotide

CTTGCCCCCAGAATGGAGGAGGATGCGCAGGTGTCTGTmod GAACGGGGGTCTTACCTCCTCCTACGCGTCCACAGACA

Bcg I nnnnnnnnnnnnnCGAnnnnnnTGCnnnnnnnnnnnn nnnnnmnnnnnnGCTnnnnnACGnnnnnnnnnnnn Cleave with Bcg I



Digest with Fok I and N.BstNB

GAACGGGGGTCTTACCTCTCTCAGCCTACCCACAGACATAATGACCCGCTCCA... CTTGCCCCCAGAATGGAGGAGA&TCGGATGGGTQTCTGTATTACTGGGCGAGGT..

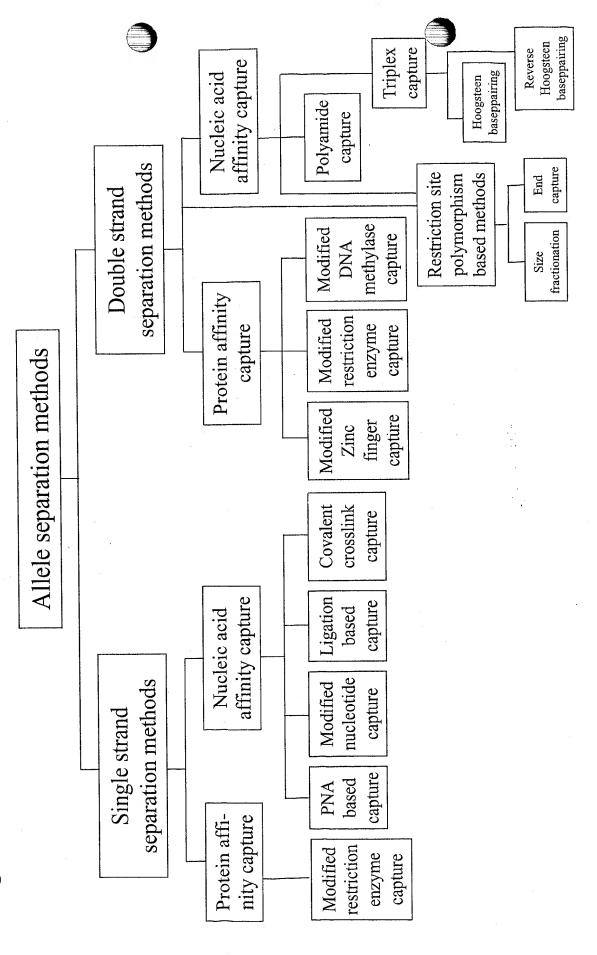
CTTGCCCCCAGAATGGAGGGAGAGTCGGAT GGGTGTCTG $^{10}$  ATTACTGGGCGAGGT... GACCCGCTCCA.. GAACGGGGGTCTTACCTCCTCAGCCTACCCACAGACATAAT

GTGGCGAACGGGGGTCTTACCTCC-CACAGACATAATGACCCGCTCCA... CTTGCCCCCAGAATGGAGGA GrĞŤGŤCTG ...CACCGCTTGCCCCCAGAATGGAGGAGG-GTGTCTG 7ATTACTGGGCGAGGT.. CTTGCCCCCAGAATGGAGGATGGrG1|dtc1GTATTACTGGGCGAGGT. GAACGGGGGTCTTACCTCCTACC-CACAGACATAATGACCCGCTCCA... **Polymorphic** nucleotide Fok I Loop ribonucleotide PCR amplify Deleted base Fok I Target Sequence Primer

Digest with Fok I and cleave with base

CTTGCCCCCAGAATGGAGGATGGrG TGTCTGT ATTACTGGGCGAGGT... GAACGGGGGTCTTACCTCCTCCTACC-CACAGACATAAT GACCCGCTCCAC...

Figure 11. Methods for haplotyping based on physical allele separation



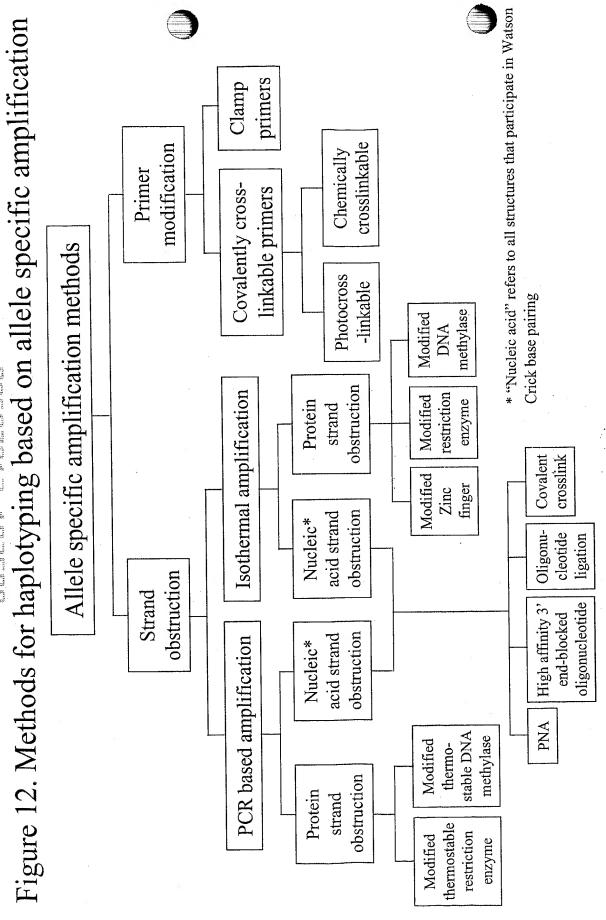
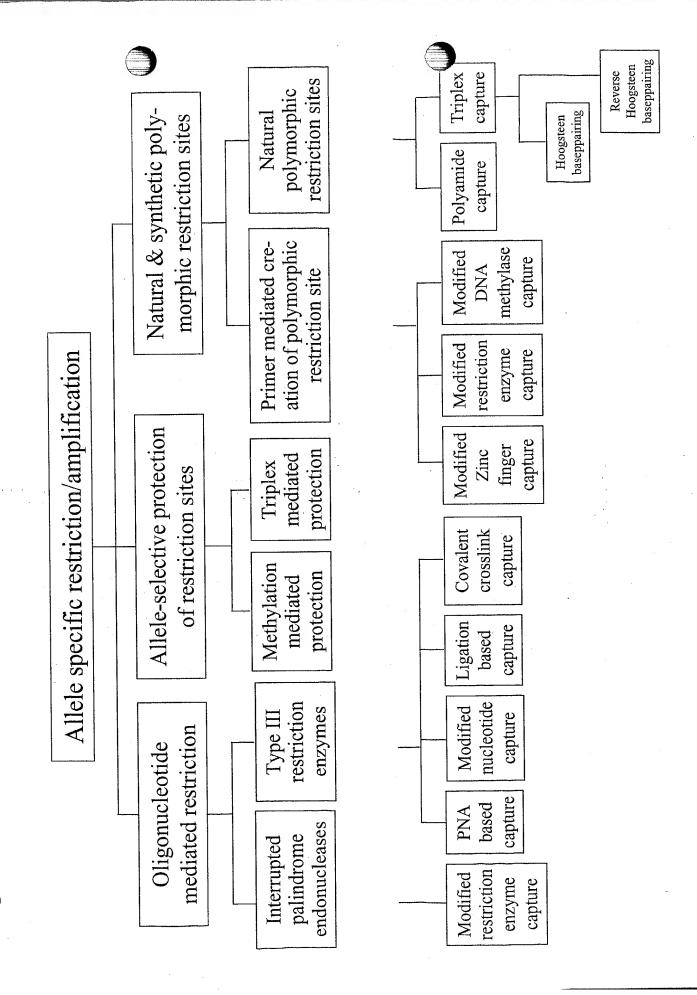


Figure 13. Methods for haplotyping based on allele specific restriction



## DCD Drimers

Figure 14	Figure 14: Hairpin Fun Filmers
ATCTGGANNN	ATCTGGANNNNNNNNNTCC
ALLELE 1 T PRIMER	→ AGGTCTA PCR Amplify
ATCTGGANNNI TAGACCTNNNI	ATCTGGANNNNNNNNNNNTCCAGAT TAGACCTNNNNNNNNNNAGG7CTA
ATCTGGANNN	ATCTGGANNNNNNNNNNTCC AGGCCTA
ALLELE 2 T PRIMER	PCR Amplify
ATCTGGANNN	ATCTGGANNNNNNNNNNNTCCGGAT TAGACCTNNNNNNNNNNNAGGCCTA TAGACCTNNNNNNNNNNNAGGCCTA

ATCCGGAI C PRIMER ATCCGGAI TAGGCCT	ATCCGGANNINNINNINNINNINNINNINNINNINNINNINNINNI	
ALLELE 2 C PRIMER  ATCCGGA ALLECE 2 ATCCGGA TAGGCCT	ATCCGGANNINNINNINNINNINNINNINNINNINNINNINNINNI	

## Figure 16: Hairpin PCR Primers

as strand resulting from PCR of allele 1
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St
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Minus strand re-
$\sum_{i=1}^{n}$

Minus Strand

TAGACCTINININININININININAGGTCTA

Hairpin loop forms inhibiting hybridization of PCR primer and amplification of allele 1

Minus Strand

N AGGTCTA -N N N<sup>N</sup>

T PRIMER

ALLELE 1

N N N N

Minus Strand

Minus strand resulting from PCR of allele 2

TAGACCTININININININININIAGGCCTA \_\_\_\_\_

ALLELE 2 T PRIMER

N N N N TCC GAT
N AGGCCTA -

PCR primer and amplification of allele 2

Minus Strand

mismatch allowing hybridization of

Hairpin loop doesn't form due to

## Figure 17: Hairpin PCR Primers

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and
Str
Minus strand resulting from PCR of allele 1
Mir
=

Minus Strand Hairpin loop doesn't form due to TAGGCCTINININININININIAGGTCTA

N N N N C GAT
N AGGTCTA --N N N N N N

C PRIMER

ALLELE 1

Minus Strand

PCR primer and amplification of allele 1

mismatch allowing hybridization of

Minus strand resulting from PCR of allele 2

Minus Strand Hairpin loop forms inhibiting hybridization of PCR primer TAGACCTININININININININAGGCCTA

ALLELE 2 C PRIMER

N N N N TCCGGAT
N AGGCCTA --

and amplification of allele 2

Minus Strand



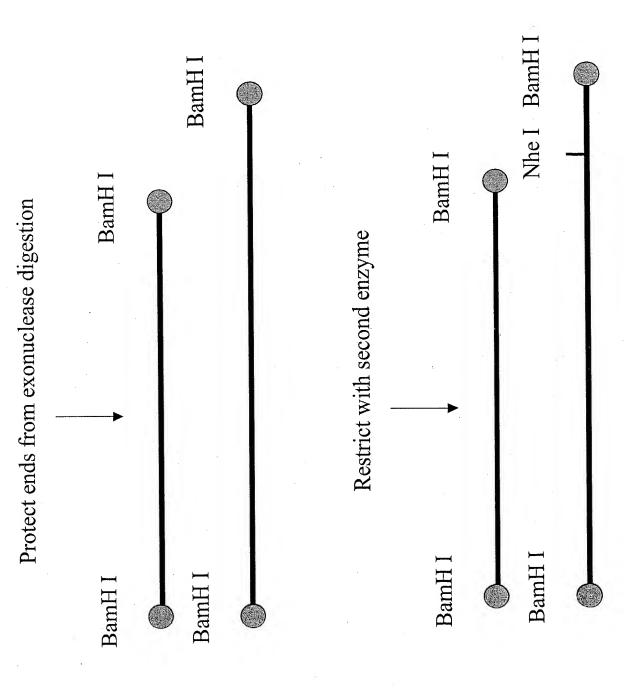


Figure 20

Digest with exonuclease

Add single strand nuclease to remove/degrade remaining single strand

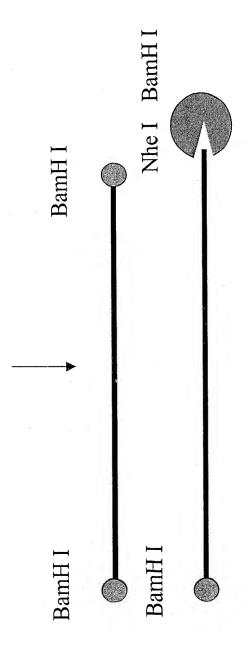
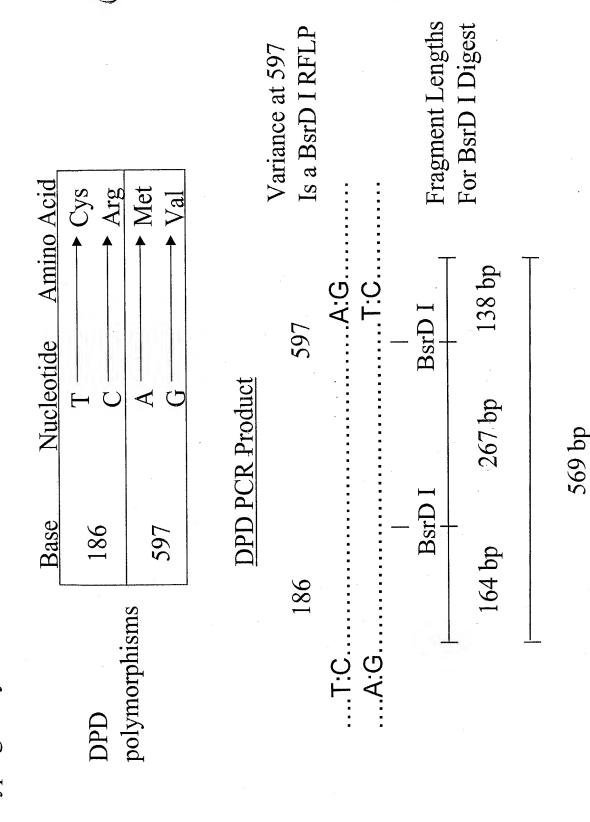


Figure 21. Dihydropyrimidine dehydrogenase (DPD) polymorphisms used in haplotyping assay.



## Figure 22. Allele Specific Primers for DPD

5° segmentions of the	מכנים אם פרוכם וליהום המיחור המיחו	S acq cagactcatgcaactctg	, , , , , , , , , , , , , , , , , , ,	actcatgcaactctg
יולט א רומרו	DFDASCF	DPDASTF		DPDNSF
<b>A.</b>	ממת	UPD	Primers	

tgagtacgttgagac[A or G]caaggtgaagccggttctt 5' actcatgcaactctg[T or C]gttccacttcggccaagaa DPD Sequence

B.

## Figure 23. PCR Amplification Using DPDNSF Primer

2.	2.	The state of the s	
<sup>5</sup> 'actcatgcaactctg	5'actcatgcaactctg	5'actcatgcaactctgTgttccac3,	5, actcatgcaactctgCgttccac
DPDNSF primer	DPDNSF primer	T allele	C allele
Template: T allele	Template: C allele	PCR Product	PCR Product

## dealt hard court from the first the court again court, as yet the first that the first from the

## Figure 24. PCR Amplification Using DPDASTF Primer.

2	: :	A service of the serv	
		3,	; 3, 3,
5' <u>acgcag</u> actcatgcaactctg 	5' <u>acgcagactcatgcaactctg</u> 	5, acgcagactcatgcaactctgTgttccac	5, accicadactcatgcaactctgCgttccac
DPDASTF primer Template T allele	DPDASTF primer Template C allele	T allele PCR Product	C allele PCR Product

## The state of the s

## Figure 25. PCR Amplification Using DPDASCF Primer

5.	 		on the second se
		33	: 5. 3
5'acacagactcatgcaactctg	5' <u>acacag</u> actcatgcaactctg 	5, acacagactcatgcaactctgTgttccac.	5'acacagactcatgcaactctgCgttccac
DPDASCF primer Template T allele	DPDASCF primer Template C allele	T allele PCR Product	C allele PCR Product

Figure 26. Hairpin Structures for PCR Products Generated Using DPDNSF Primer

Hairpin Structure T Allele Reverse Strand

Not Stable

Hairpin Structure C Allele Reverse Strand

 $\vdash$  gttgcatgag  $\stackrel{3'}{\vdash}$   $\vdash$  agacGcaaggtg......

 $Tm = 40^{\circ}C$ 

Figure 27. Hairpin Structures for PCR Products Generated Using DPDASCF Primer

Hairpin Structure T Allele Reverse Strand

Hairpin Structure C Allele Reverse Strand

 $Tm = 42^{\circ}C$ 

3

Figure 28. Hairpin Structures for PCR Products Generated Using DPDASTF Primer

Hairpin Structure T Allele Reverse Strand

> Hairpin Structure C Allele Reverse Strand

Figure 29. Non-Allele Specific Amplification Using DPDNSF Primer.

#### ALLELE C

**DPDNSF** primer 5' actcatgcaactctg

 $Tm = 41^{\circ}C$ 

## ALLELE T

DPDNSF primer

5'actcatgcaactctg

3

 $^{3}$ , Tm = 41 °C

agacGcaaggtg.... gttgcatgag

 $Tm = 40^{\circ}C$ 

Not Stable Primer cAcaaggtg..... agagttgcatgag

and Amplification Hybridization

and Amplification Hybridization Primer

,tgagtacgttgagacAcaaggtg... actcatgcaactctg

3

3,tgagtacgttgagacGcaaggtg... 

Ŝ

## Figure 30. Allele Specific Amplification Using DPDASCF Primer

#### ALLELE C

DPDASCF primer  $Tm = 60^{\circ}C$   $\frac{5}{acacagactcatgcaactctg}$  3

Primer
Hybridization
and Amplification

3, acacagactcatgcaactctg 3, interceptage 3, interceptage of the same of the sa

### ALLELE T

DPDASCF primer  $Tm = 60^{\circ}C$   $\frac{3}{acacag}$  actcatgcaactctg

Hairpin inhibits
Primer Hybridization
and Amplification

5' acacagactcatgcaactctg

# Figure 31. Allele Specific Amplification Using DPDASTF Primer

#### ALLELE C

DPDASTF primer  $Tm = 65^{\circ}C$ 

5' acq caq act cat g ca a ct ct g

 $= atgagtciqcqt \\ \hline | Tm = 100^{\circ}C \\ - cgttgagacGcaaggtg.....$ 

Hairpin inhibits primer hybridization and Amplification

5'acgcagactcatgcaactctg

### ALLELE T

DPDASTF primer Tm = 65°C

acqcaqactcatqcaactctg

cgttgagacAcaaggtg.....

Primer hybridizes and amplification ensues

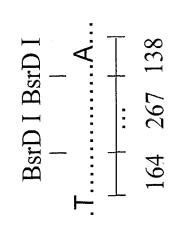
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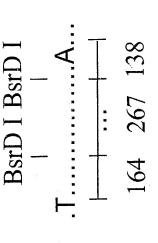
Figure 32. Allele Specific Amplification of a Heterozygous Sample with Haplotype T<sup>186</sup>, A<sup>597</sup> and C<sup>186</sup>, G<sup>597</sup>

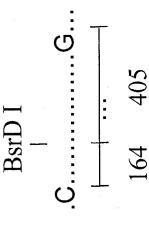
## DPDNSF PRIMER

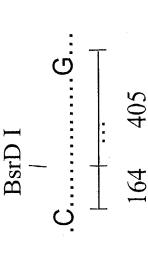
## DPDASTF PRIMER

## DPDASCF PRIMER









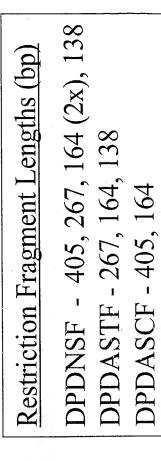
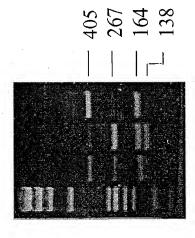


Figure 33. BsrD I Digest of Allele Specific PCR Products.

DPDASCF DPDASTF DbDNSE Phi X 174 Hae III



T G C Fok I/Fsp I
A G C Loop
CCCGGCTGGGCGCGGACATG AGGACGTG

GCAGGCCCGGCTGGGCGCGCGGACATGGAGGACGTGTGCGGCCGCCTGGTGCAGTACCGC CGTCCGGGCCGACCCGCGCCTGTACCTCCTGCACGCCGGCGGGGGCGCACGTCATGGCG

Target Sequence

GECGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAGGAGCTGCGGGTGCGCCTCGCCT CCGCTCCACGTCCGGTACGAGCCGGTCTCGTGGCTCCTCGACGCCCACGCGGAGCGGA

Target Sequence

GGTGGACGCGTTCGACGCATTCGCCGAGGAGGCGCTACGGCTACTGGACGTCTTCG
GGCTACTGGACGTCTCG CCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGCGATGCCGATGACCTGCAGAAGC

ApoE21250-LR

T Allele Amplicon

CCCGGCTGGGCGCGGACATGGGATGCGCAAGGACGTGTGCGGCCGCCTGGTGCAGTAC GGGCCGACCCGCGCCTGTACCCTACGCGTATCCTGCACACGCCGGGCGGACCACGTCATG CGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAGGAGCTGCGGGTGCGCCTCG GCGCCGCTCCACGTCCGGTACGAGCCGGTCTCGTGGCTCCTCGACGCCCACGCGGAGC

GGAGGTGGACGCGTTCGACGCATTCGCCGAGGCGCTACGGCTACTGGACGTCTTCG CCTCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGCGATGCCGATGACCTGCAGAAGC

#### C Allele Amplicon

CCCGGCTGGGCGCGCATGGGATGCGCAAGGACGTGCGCGGCCGCCTGGTGCAGTAC  CGCGGCGAGGTGCAGGCCATGCTCGGCCAGAGCACCGAGGAGCTGCGGGTGCGCCTCG GCGCCGCTCCACGTCCGGTACGAGCCGGTCTCGTGGCTCCTCGACGCCCACGCGGAGC

GGAGGTGGACGCGTTCGACGCATTCGCCGAGGCGCGCTACGGCTACTGGACGTCTTCG CCTCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGCGATGCCGATGACCTGCAGAAGC